Investigation of acute effects of graphene oxide on wastewater microbial community: A case study

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ABSTRACT

The market for graphene-based products, such as graphene oxide (GO), is projected to reach nearly $675 million by 2020, hence it is expected that large quantities of graphene-based wastes will be generated by then. Wastewater treatment plants will be one of the ultimate repositories for these wastes. Efficient waste treatment relies heavily on the functions of diverse microbial communities. Therefore, systematic investigation of any potential toxic effects of GO in wastewater microbial communities is essential to determine the potential adverse effects and the fate of these nanomaterials in the environment. In the present study, we investigate the acute toxicity, i.e. short term and high load, effect of GO on the microbial functions related to the biological wastewater treatment process. The results showed that toxic effects of GO on microbial communities were dose dependent, especially in concentrations between 50-300 mg/L. Bacterial metabolic activity, bacterial viability, and biological removal of nutrients, such as organics, nitrogen and phosphorus, were significantly impacted by the presence of GO in the activated sludge. Furthermore, the presence of GO deteriorated the final effluent quality by increasing the water turbidity and reducing the sludge dewaterability. Microscopic techniques confirmed penetration and accumulation of GO inside the activated sludge floc matrix. Results demonstrated that the interaction of GO with wastewater produced significant amount of reactive oxygen species (ROS), which could be one of the responsible mechanisms for the toxic effect of GO.

KEYWORDS: Graphene oxide, microbial community, toxicity, wastewater treatment

1. INTRODUCTION

Graphene oxide (GO) is the functionalized form of graphene containing epoxy, hydroxyl, and carboxyl groups [1, 2]. GO possesses excellent electrochemical properties, hence, it has wide applications in electronics, biosensors, pipes, semiconductor, and packaging in both pure and nanocomposite forms [3, 4]. Due to the potential wide utilization of this nanomaterial, it is expected that wastes containing this nanomaterial will be generated and end up in landfills and wastewater treatment plants.

A typical wastewater treatment plant utilizes the functions of diverse groups of microorganisms for degradation of organic matter, remediation of toxic or carcinogenic compounds and removal of excess nutrients (nitrogen and phosphorus) to reduce the pollution of receiving waters [5]. However, contaminants in the wastewater influent may adversely affect the functions of these microorganisms. In recent years, disposal and fate of nanomaterials in aquatic systems have become a matter of concern; however very few studies are available on this topic. One study recently demonstrated that high loads (219 mg/L) of single walled carbon nanotubes (SWNT) can differentially impact various microbial communities in activated sludge processes and adversely affect treatment efficiency [6].

In the case of GO, no studies so far have investigated the effects of GO on the wastewater processes. However, the fact that SWNT, like GO, is also made of graphene and presents adverse effects to activated sludge processes, it is reasonable to hypothesize that GO will also present toxic effects to the wastewater microbial community. Additionally, several studies have reported that better dispersion and longer contact time of carbon-based nanomaterials with pure bacterial cultures increase their antimicrobial effects [7-10]. When comparing GO to SWNTs, GO presents stronger hydrophilic nature and is more stably dispersed in aqueous solution than SWNTs. Hence these properties can potentially enhance the contact of GO with microbial

communities and produce stronger adverse effects to the wastewater treatment process than SWNTs.

Furthermore, in recent studies investigating the antibacterial properties of GO to pure bacterial cultures, it was demonstrated that GO is toxic to pure bacterial cultures (Gram-positive and Gram-negative) on both planktonic and biofilm stages [11-13]. In these studies, depending on the concentrations (~40-80 mg/L), significant levels of inactivation (~60-80%) were observed in pure cultures [12-14]. So far, all these studies on the antimicrobial properties of GO were done using microorganisms in pure cultures under controlled laboratory conditions. However, natural and engineered aquatic systems are more complex than the simplified system used in these studies in terms of microbial community, solution chemistry, nanomaterial aggregation, and presence of suspended particles and natural organic matter. Therefore, more complex environments need to be investigated to determine the real impact of GO to the environment and its effect on the normal functions of the ecosystem [11, 12].

The global market for graphene-based products, such as graphene, is projected to increase in 5 years in 51.7% and reach a global market of $122.9 M in 2017 and $986.7 M in 2022 [4]. Therefore, wastewater treatment plants can potentially experience an acute exposure to this nanomaterial, i.e. short term exposure to high GO concentrations (ppm level). The objective of this study is to evaluate the effects of acute exposure of wastewater microbial communities to GO, using batch scale tests. The short term effects of GO on wastewater microbial communities were evaluated in terms of metabolic activity and bacterial inactivation. The effects of GO on wastewater process performance were evaluated through bacterial removal of organic carbon (Biochemical Oxygen Demand, BOD), removal of nutrients (ammonia nitrogen, NH_3-N and phosphate, PO_4^-), effluent quality (turbidity), and sludge quality (dewatering properties).

2. MATERIALS AND METHODS

2.1. GO Preparation and Characterization

GO was prepared by the modified Hummers method [15]. All chemicals were reagent grade and were purchased from Sigma-Aldrich and Fisher Scientific, USA. Briefly, graphite flakes (Alfa Aesar, USA) were allowed to react with $NaNO_3$ and concentrated H_2SO_4 for 30 min in ice bath. Later, oxidation was carried out by adding $KMnO_4$ and incubating at 35 °C for 12 h. Further oxidation was carried out by adding H_2O_2 at 90 °C in oil bath. The resultant mixture was sieved out with 425 and 250 µm US Standard Testing Sieves to remove any remaining graphite flakes. The resultant solution was centrifuged repeatedly with base and acid washing steps to neutralize pH to 7.0. The GO pellet was collected and washed with methanol and dried for 3 days in a vacuum oven. Then, the dried GO pellets were suspended in deionized water (DI) to prepare GO stock solutions with a concentration of 500 mg/L and were homogenously dispersed by probe sonication (5 min) (Tekmar, USA) and bath sonication (24 h). Prior to the toxicity assays, the stock solution was vortexed for a few seconds.

The characterization of the prepared GO was carried out using atomic force microscopy (AFM) to examine morphology and X-ray photoelectron spectroscopy (XPS) to determine the functional groups. The topographical measurement of the nanomaterial was done under ambient conditions with a PicoSPM II (PicoPlus, Molecular Imaging-Agilent Technologies) using the intermittent contact mode. The GO sample used for AFM measurement was a spin coated GO film onto indium tin oxide (ITO) substrate.

XPS measurements were performed using a PHI 5700 X-ray photoelectron spectrometer equipped with a monochromatic Al K X-ray source (h=1486.7 eV) incident at 90° relative to the axis of a hemispherical energy analyzer. The spectrometer was operated both at high and low

resolutions with pass energies of 23.5 eV and 187.85 eV, a photoelectron take off angle of 45 ° from the surface, and an analyzer spot diameter of 1.1 mm. The survey spectra were collected from 0 to 1400 eV, and high resolution spectra were obtained for photoelectrons emitted from C1s and O1s. All spectra were collected at room temperature with a base pressure of 1 x10 $^{-8}$ torr. Electron binding energies were calibrated with respect to the C1s line at 284.5 eV (C-C). A PHI Multipak software (version 5.0A) was used for all data processing. The high resolution data were analyzed first by background subtraction using the Shirley routine and a subsequent non-linear fitting to mixed Gaussian-Lorentzian functions. Complete characterization of the synthesized GO is presented in the Supporting Information section (Figure S2 and S3).

2.2. Wastewater Sample Collection and Preparation

Activated sludge samples were collected from the aeration tank of Sims South Bayou Wastewater Treatment Plant (Houston, TX). This treatment plant uses conventional activated sludge process (Supporting Information, Figure S1) with no enhanced phosphorus or nitrogen removal process. Briefly, fresh activated sludge samples were collected and transported to the laboratory inside a styrofoam container filled with ice packs to maintain the samples at 4^{o}C. The collection and preparation of the wastewater samples were adapted from a previous study [16]. Briefly, activated sludge samples were aerated for 1 h and 20 ml was transferred to conical tubes (Corning, USA), which were used as batch reactors. Appropriate volumes of the GO stock solution were calculated and added in each reactor to attain final concentrations of 10, 20, 50, 100, 200 and 300 mg/L of GO. The GO concentrations selected for this study were based on a preliminary metabolic assay study with the activated sludge (data not shown) and on previous studies investigating acute effects of heavy metals and other nanoparticles in activated sludge. In these studies, the concentrations used were between 1 to 3000 mg/L [6, 17-19]. The incubation

was carried out at room temperature in a shaker at 200 rpm (New Brunswick Scientific, USA). To evaluate short term toxic effects, the incubation time was ~5 hr according to previous similar studies [6, 18]. All the tests were performed in triplicates and a paired t-test statistical analysis was performed. To account for daily variations in the activated sludge properties, three sludge samples were collected in different days and triplicate analyses were performed with each collected sample. The physiochemical characteristics of the wastewater were measured according to the Standard methods and presented in Table 1[5].

Table 1. Physico-chemical characteristics of the activated sludge samples based on triplicate samples collected in three different weeks.

Total suspended solids, TSS (mg/L)	2666.6±942.8
Dissolved oxygen, DO (mg/L)	9.81±0.83
pH	7.3±0.37
Ammonia-nitrogen, NH_3-N (mg/L)	1.46±0.35
Phosphate, PO_4^- (mg/L)	5.3±0.56

2.3. Bacterial Metabolic activity and Viability Assay

The microbial metabolic activity assay was performed with the activated sludge after interaction with GO according to previously reported procedure [20]. Briefly, triplicates of 100 µl of the activated sludge were exposed to different concentrations of GO for 5 h in a 96-well flat bottom plate (Corning, USA). A volume of 60 µl (based on calibration curve, Figure S4) of C_{12}-resazurin (Vybrant Cell Metabolic Assay kit, Molecular Probe, USA) was added to each well. The mixtures in the 96-well plates were incubated for 15 minutes at 37 °C in the dark. In the presence of metabolic active cells, C_{12} resazurin is reduced to red fluorescent C_{12}-resofurin. The production of C_{12}-resofurin by the activated sludge was quantified with a Synergy MIX Microtiter plate reader (BioTek, USA) at 530/587 nm wavelength. Bacterial viability test was

done according to the heterotrophic plate count agar methodology described in the Standard Methods [5].

2.4. Organic carbon degradation test

A Biochemical Oxygen Demand (BOD_5) test was performed according to the Standard Methods to investigate the bacterial capacity to degrade organic carbon in presence of GO [5]. Briefly, after 5 h incubation, 15 ml of samples from each reactor were placed and mixed in 300 ml BOD bottles. A DO probe with stirrer (YSI Incorporated) was used to read the initial DO and final DO (after 5 d incubation).

2.5. Removal of nutrients (Nitrogen and Phosphorus)

Removal of Nitrogen was measured by determining the conversion of ammonia (NH_3-N) to nitrate (NO_3^-) (nitrification process), while removal of phosphorus was measured in terms of phosphate (PO_4^-) bacterial uptake. Soluble ammonia-nitrogen (NH_3-N), NO_3^- and PO_4^- were measured from the incubated samples according to previously described methods [5, 21]. Since nitrification is a slow process (~20 h), GO and activated sludge samples were incubated for 20 h prior to ammonia and nitrate final concentration measurements. These tests were done as follows: 5 ml of the control and the incubated activated sludge samples with GO were filtered through 0.22 µm membrane filters to remove the suspended flocs and 1 ml of the filtrate was diluted with DI water for ammonia, nitrate and phosphate quantifications. Colorimetric methods were used to quantify NH_3-N, NO_3^- and PO_4^- in the filtrate with a DR3900 spectrophotometer (Hach, USA). NH_3-N, NO_3^- and PO_4^- were measured by the salicylic acid method (method 8155, Hach), cadmium reduction method (method 8039, Hach) and the ascorbic acid method (method 8048, Hach), respectively.

2.6. Effluent quality and sludge dewatering property

To determine the effluent quality and sludge dewatering ability, turbidity of the supernatant (nephelometric turbidity unit, NTU) and capillary suction time (CST) were measured, respectively, after the settling of the activated sludge. Both the NTU and CST were measured according to the Standard Methods [5]. Briefly, after 5 h incubation with GO, batch reactors with activated sludge samples were left to settle for a period of 2 h, which is the average residence time in the clarifier. The supernatant was removed carefully for turbidity measurement, while the settled sludge was used for CST measurement. Briefly, for the CST measurement, a stainless steel tube with inner radius of 1.5 inch was placed on a coarse type filter paper (Whatman, UK) and a 5 inch radius circle was drawn around the tube on the filter. Settled sludge (2 ml) samples were quickly released inside the tube and the time to wet the filter from radius 1.5 to 5 inch was recorded as CST.

2.7. Scanning electron microscopy (SEM) and Fluorescence imaging

To observe the interaction of activated sludge flocs and microorganisms with GO, SEM and fluorescence microscopy were conducted [10, 16, 22]. Briefly, for the SEM samples, at the end of 5 h incubation, 0.5 ml of solution was taken from each reactor and fixed with 2% gluteraldehyde in 0.05M cacodyle buffer solution (Fisher Scientific, USA). Fixed samples were serially dehydrated with increasing concentrations of ethanol (25%, 50%, 75%, 95% and 100%). SEM images were acquired at 10 Kev accelerated voltage with JSM 6010LA (Jeol, USA). For fluorescence imaging, 0.2 ml of each sample was stained with 0.2 µl of green dye (SYTO9, Invitrogen, USA) and images were taken with a fluorescence microscope (OLYMPUS, Japan).

For each sample, 10 representative images were recorded. GO dispersion in DI water was imaged under bright field condition with a fluorescence microscope (OLYMPUS, Japan).

2.8. Reactive Oxygen Species (ROS) production and oxidative stress

Dose dependent ROS production by GO was reported in previous studies [23]. ROS are known to cause oxidative stress in cells, hence ROS production by GO in wastewater was investigated [8, 23, 24]. ROS production was quantified as oxidation of glutathione (GSH) according to Ellman's Assay method described elsewhere [24]. GSH is a thiol containing polypeptide present in prokaryotic and eukaryotic cells which is known to protect the cells from stress caused by ROS [23-25]. Oxidation of GSH in aqueous solution in presence of nanomaterials is an indirect measure of ROS production. Briefly, various GO concentrations in wastewater were filtered and were spiked with GSH in bicarbonate buffer solution in a 12 well-plate. Then, the plate was incubated for 2 h in dark to prevent any photochemical reaction. After the incubation period, the Ellman's reagent, 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), was added and the resultant yellow solution was filtered with a 0.22 µm filter to remove GO from the solution. DTNB reacts with aqueous GSH which can be quantified colorimetrically. A volume of 200 µL of the solution was placed in a 96-well plate and quantified at 412 nm wavelength with the Synergy MIX Microtiter plate reader (BioTek, USA). The negative control did not contain GO, whereas in the positive control contained 1 mM of H_2O_2 for oxidation of the GSH.

3. RESULTS AND DISCUSSIONS

3.1. Effects on bacterial metabolic activity and viability

The acute toxicity effects of GO was first evaluated through the metabolic activity assay of the wastewater bacterial community in the presence of different concentrations of the nanomaterial. In this assay, only viable bacteria are able to reduce non-fluorescent resazurin to red-fluorescent

resofurin [20]. The results of this assay showed that, at all GO concentrations tested, significant inhibition of the wastewater microbial community metabolic activity (~20-70%) was observed (Figure 1). Additionally, the results show that the toxicity of the nanomaterial is concentration dependent, since at higher GO concentrations (100-300 mg/L), significantly higher inhibition of bacterial metabolic activity (~50-70%) was observed. The GO concentration at 300 mg/L showed statistically significant inhibition of metabolic activity compared to the other GO concentrations. These results agree with several toxicity studies with bacterial pure cultures, where significant inhibition of the bacterial metabolic activity was observed in the presence of GO and other carbon-based nanomaterials (e.g. carbon nanotubes and fullerenes) [8, 11, 12, 26].

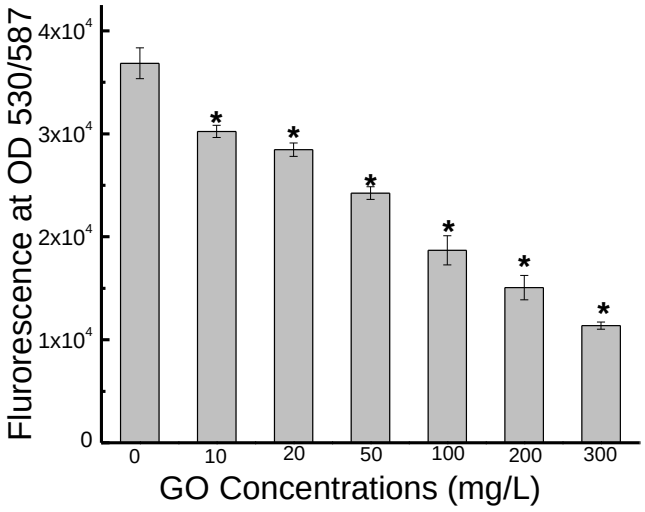

Figure 1. Metabolic activity of the bacteria in activated sludge after 5 h incubation with different concentrations of GO. ***** refers to statistically significant different results between control and the corresponding sample. Control sample does not contain any nanomaterial.

The antibacterial effect of GO in wastewater was further verified by the plate count method. The results from the plate counts corroborated the metabolic activity assay, since the increase in GO concentrations resulted in reduced numbers of viable bacteria (Figure 2).

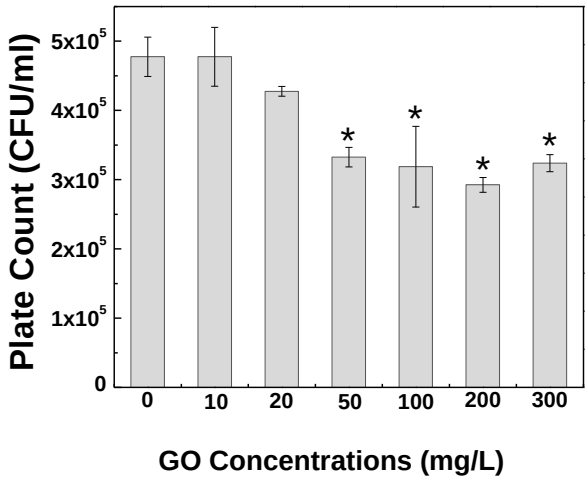

Figure 2. Enumeration of total viable bacterial cells (CFU/ml) in activated sludge after 5 h incubation with different concentrations of GO. ***** refers to statistically significant different results between control and the corresponding sample. Control sample does not contain any nanomaterial.

The significant reduction of metabolic activity (~50-70%) at GO concentrations of 100-300 mg/L, resulted in ~35% bacterial growth inhibition. This difference in the bacterial growth inhibition compared to bacterial metabolic activity was previously observed in antibacterial studies with other carbon-based nanomaterials [8]. This difference was explained by the fact that bacterial cells will, under unfavorable conditions, reduce their metabolic activity and resume growth when switched to favorable conditions (i.e., addition of nutrients or removal of an inhibitor), like a bacteriostatic agent [8, 27]. This reduced bacterial metabolic activity and viability results suggest that GO can potentially inhibit the essential biological functions of bacteria in the activated sludge process.

3.2. Inhibition of biodegradation of organic carbon

In order to verify the acute effect of GO in the wastewater treatment process, a standard BOD_5 test was conducted to determine the ability of microorganisms to remove the organic matter in

the wastewater under aerobic conditions. The results showed > 50% reduction in the BOD_5 at all concentrations of GO (Figure 3). These results can be explained by the lower bacterial metabolic activity observed in the metabolic activity assay (Figure 1). During the BOD_5 tests, the lower metabolic activity of microorganisms in the presence of the nanomaterials led to reduced oxygen consumption by aerobic microorganisms during the metabolization of the organic waste in the wastewater, hence leading to a reduction in the BOD_5 values. It is noteworthy that the different GO concentrations in the activated sludge did not show considerable variation in their effect on the BOD values, which suggests that the minimum concentration to inhibit degradation of organic matter is around 10 mg/L. In a similar study with multi-walled carbon nanotubes (MWNT), ~50 % BOD reduction was observed at various concentrations. However, with MWNT, the concentrations used were much higher (1440 to 3240 mg/L) than the GO concentrations used in this study [28].

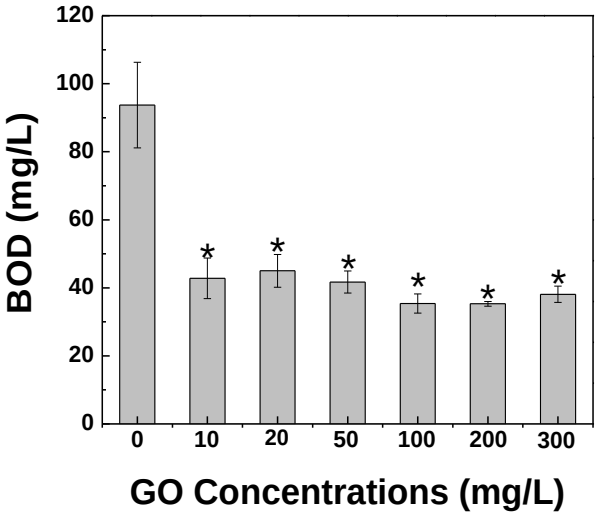

Figure 3. 5-day BOD results of the activated sludge samples with different concentrations of GO. ***** refers to statistically significant different results between control and the corresponding sample. Control sample does not contain any nanomaterial.

3.3. Effects of GO on the biological process of Nitrogen removal and Phosphorus accumulation

Nitrogen (as NH_3-N) and phosphorus (as PO_4^-) are two major nutrients that must be removed from the influent during wastewater treatment. The microbial communities in the activated sludge responsible for removing nitrogen and phosphorus are ammonia oxidizing bacteria (AOB) and polyphosphate accumulating organisms (PAO), respectively. Details of the chemical processes of nitrogen and phosphorus removal are described in the Supporting Information section. Briefly, in a functional activated sludge process, ammonia is converted to nitrate, through the aerobic process of nitrification. In our study, with increasing GO concentrations, we observed ammonia accumulation due to reduced conversion of ammonia to nitrate (Figure 4), which suggests inhibition of nitrifying bacteria in the activated sludge sample in the presence of GO.

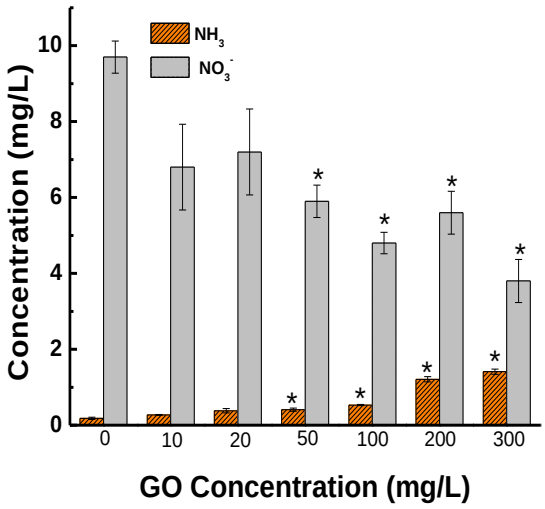

Figure 4. Concentrations of NH_3-N and NO_3^-, measured in activated sludge samples incubated with different GO concentrations. * refers to statistically significant different results between control and the corresponding sample. Control sample does not contain any nanomaterial.

Another important microbial community in the wastewater treatment process is the PAO community, which is responsible for removing phosphorus nutrients from the wastewater. In the wastewater process, phosphorus exists as PO_4^- which gets accumulated by PAO and hence removed from the wastewater.

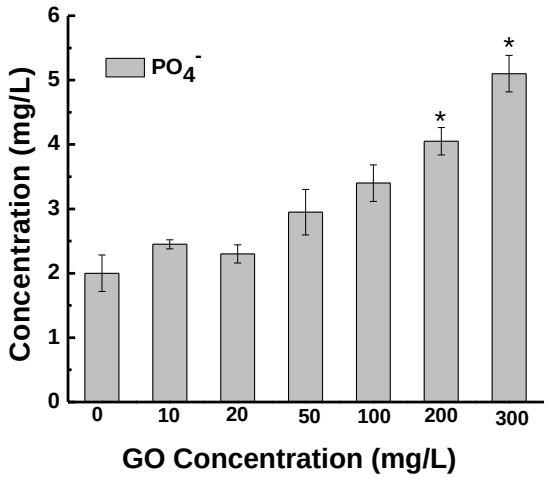

Figure 5. Concentrations of PO_4^-, measured in activated sludge samples incubated with different GO concentrations. * refers to statistically significant different results between control and the corresponding sample. Control sample does not contain any nanomaterial.

In our results (Figure 5), significant effects of GO on the PAO microbial community were observed at higher concentrations of GO (200 – 300 mg/L). At these high concentrations, the activity of PAO was inhibited since PO_4^- concentrations in the wastewater did not decrease over time as observed in the control samples.

Therefore, these results suggest that acute exposure of the wastewater microbial community to GO, especially at higher concentrations (~100-300 mg/L), can inhibit the activated sludge

microbial community functions, such as ammonia degrading and phosphate accumulating microbial communities.

3.4. ROS Production and oxidative stress

Several studies have shown that carbonaceous nanomaterials like GO can produce chemically reactive species in aqueous solution and can adversely impact microbial and eukaryotic cell structures [23-25, 29]. In our study, significantly higher ROS production was found at high concentrations of GO in the wastewater samples when compared to the control samples (Figure 6). Previous studies with GO in aqueous solution demonstrated ROS production was a dose dependent phenomena [23]. The increased loss of GSH at higher concentrations of GO samples (200 and 300 mg/L) suggests that ROS production could be contributing to the increasing toxicity observed (~60-70%, Figure 1) to the microbial community at those concentrations. Although we did not determine the exact toxicity mechanism generated by the ROS on the wastewater microbial community, other studies suggest that ROS can cause severe damages to bacterial DNA, proteins and cell membranes as a cause for GO toxicity [8, 11, 16].

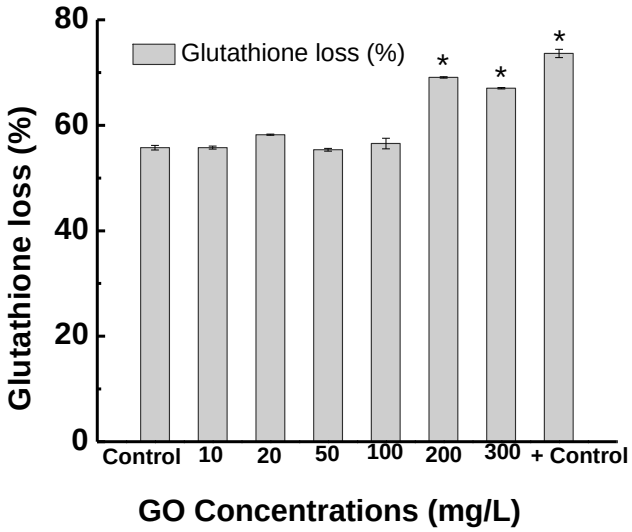

Figure 6. Loss of Glutathion as an indicator of ROS production. * refers to statistically significant different results between control and the corresponding sample. Control sample does not contain any nanomaterial. + Control contains H_2O_2 as oxidizing agent.

3.5. Effects of GO on sludge settling and dewatering

In a typical activated sludge process, turbidity of the effluent is an indicative of the effectiveness of the treatment process and successful removal of organic matter from the wastewater. High turbidity carries two implications in the disinfection process before the discharge into receiving waters. First, higher turbidity signifies higher organic matter content in the water. To treat such wastewater, higher concentrations of disinfecting agent (chlorine) will be required, since organic matter can reduce the availability of free chlorine for microbial disinfection [30]. Secondly, the presence of organic matter increases formation of carcinogenic disinfection by-products (DBP), since chlorine reacts with organic matter to produce DBP [31]. Therefore it is important to determine the effect of GO on the turbidity of the effluent. In a typical wastewater treatment plant, turbidity of the supernatant of the settled sludge in a clarifier unit is routinely measured to monitor the effectiveness of the treatment process. We performed similar settling tests in the batch reactors to observe any potential effects of GO on the turbidity of the supernatants.

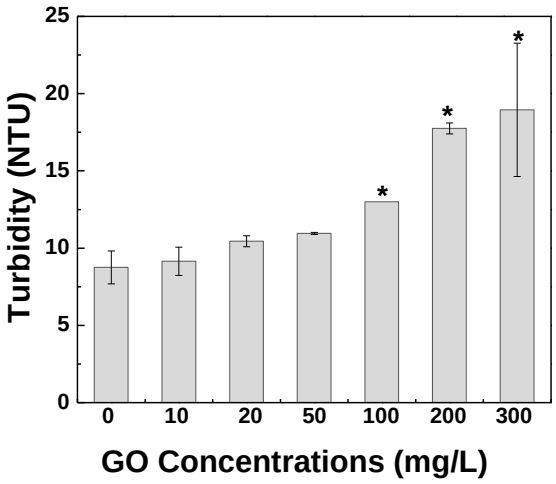

Figure 7. Turbidity (NTU) of the supernatant of activated sludge samples after 2 h settling period.* refers to statistically significant different results between control and the corresponding sample. Control sample does not contain any nanomaterial.

The results of the sludge settling test showed that with the increase in GO concentrations, the turbidity of the effluent steadily increased (Figure 7). Microscopic analysis of the supernatant revealed that increased turbidity is attributed to the presence of both suspended GO and attached organic matters onto GO surfaces (Figure S5). Overall it was observed that the addition of GO to the wastewater effluent increased the final effluent turbidity. The turbidity in the presence of GO was higher than the recommended value of 5 NTU for wastewater effluent discharge.

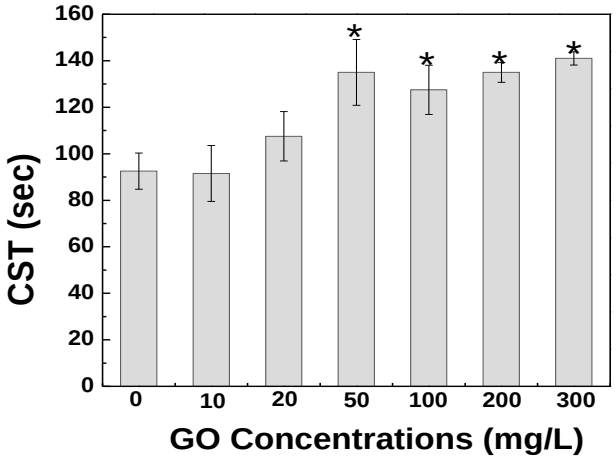

Figure 8. Capillary suction time (CST) required for dewatering of settled activated sludge samples after 2 h settling period. * refers to statistically significant different results between control and the corresponding sample. Control sample does not contain any nanomaterial.

The sludge dewatering, or removing water from the sludge, is the final process in the wastewater treatment. The dewatering of sludge is very important since dewatered solids are cheaper and easier to incinerate, produce less offensive smells, and reduce volume and disposal costs in landfills [32]. The typical values of CST for the municipal sludge dewatering are variable, but usually are >100 seconds, these values are highly dependent on the sludge composition and treatment steps (addition of polymer or other thickeners) [32]. In the present study, the results of the dewatering tests showed that with increasing GO concentrations, the dewatering capacity of the activated sludge is significantly reduced, since the capillary suction time (CST) increased. Increase of dewatering time was observed to be ~45% at GO concentrations of 50 mg/L and above compared to controls (Figure 8). The possible cause for this phenomenon could be a combination of both chemical reactivity and antimicrobial characteristics of GO. However these potential mechanisms need to be further investigated.

3.6. Interaction of GO and Activated sludge

The fluorescence images from the sludge samples incubated with GO shows that GO nanosheets accumulated inside the floc matrix (Figure 9c). SEM images show adsorption of bacteria and other microorganisms to the GO nanosheets (Figure 9f). Several studies suggested that the accumulation of nanomaterial in activated sludge flocs could result into longer retention of GO in the treatment system and therefore pose chronic toxicity [16]. Future studies are needed to better understand the effects of GO accumulation in the activated sludge flocs as well as the potential chronic toxicity of this nanomaterial to the microbial community.

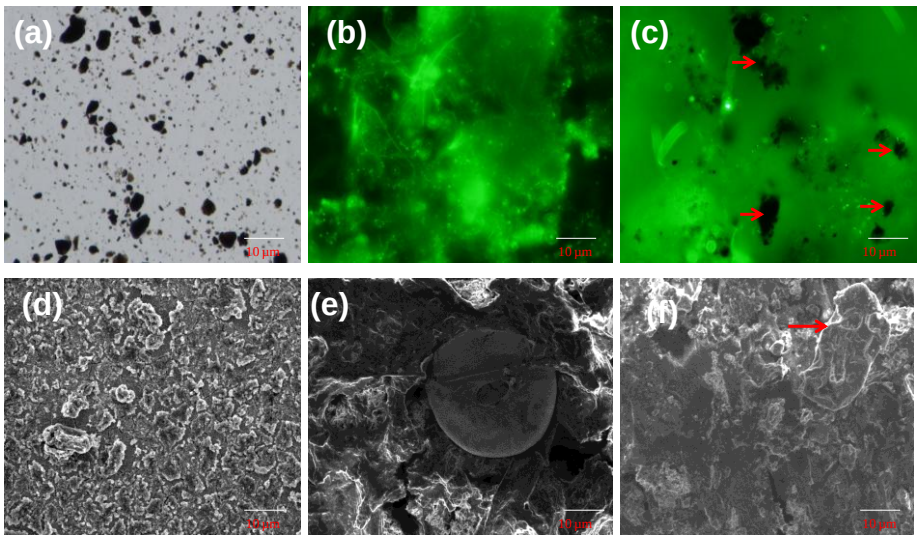

Figure 9. (a) Bright field image of aqueous suspension of GO; (b) fluorescence image of control activated sludge (no GO); (c) fluorescence image of activated sludge with GO; (d) SEM image of aqueous suspension of GO; (e) SEM image of control sludge (without GO; (f) SEM image of activated sludge with GO. Fluorescence and SEM imaging were done at 40x and 1000x magnifications, respectively. Red arrow sign indicates GO sheets in sludge flocs.

4. Conclusion

This study shows that acute exposure of activated sludge to GO can impact the wastewater microbial communities. Bacterial metabolic activity was significantly compromised in the presence of GO, which indicates that GO has the potential to hinder the essential microbial functions needed in activated sludge processes, such as removal of organic matter and other nutrients from the wastewater. The presence of GO in the activated sludge led to reduced BOD_5 values and low nitrogen and phosphorus removal by the biological treatment process, which can potentially lead to excess of organic matter, nitrogen and phosphorus, respectively, discharge

into receiving waters from the treatment plants. Furthermore, GO also negatively impacted the effluent quality and sludge dewaterbility, which can cause regulatory violations and increased disposal cost of sludge, respectively. As applications and disposal of engineered nanomaterials, such as GO, are in rapid rise, these findings suggest that further studies, especially on the chronic exposure of these nanomaterials to the wastewater microbial community is needed. Both acute and chronic microbial exposures to GO are essential for a complete understanding of the effects of GO to the wastewater treatment process and prevention of their adverse effects to the treatment performance.

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Table(s)

Investigation of acute effects of graphene oxide on wastewater microbial community: A case study

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Table 1. Physico-chemical characteristics of the activated sludge samples based on triplicate samples collected in three different weeks.

Total suspended solids, TSS (mg/L)	2666.6±942.8
Dissolved oxygen, DO (mg/L)	9.81±0.83
pH	7.3±0.37
Ammonia-nitrogen, NH_3-N (mg/L)	1.46±0.35
Phosphate, PO_4^- (mg/L)	5.3±0.56

Source File

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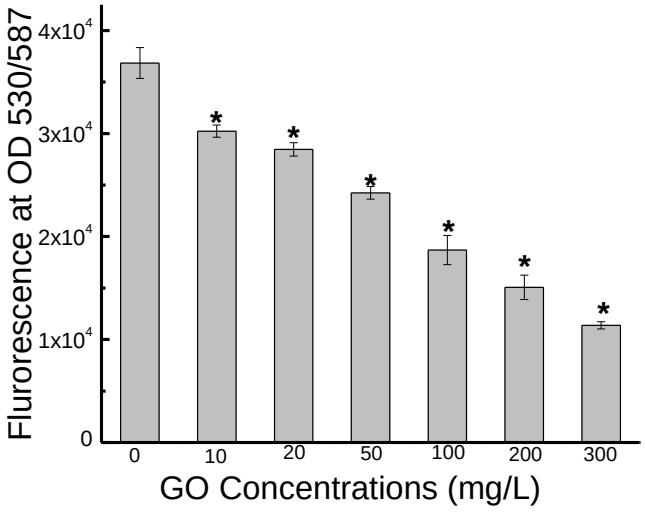

Figure 1. Metabolic activity of the bacteria in activated sludge after 5 h incubation with different concentrations of GO. ***** refers to statistically significant different results between control and the corresponding sample. Control sample does not contain any nanomaterial.

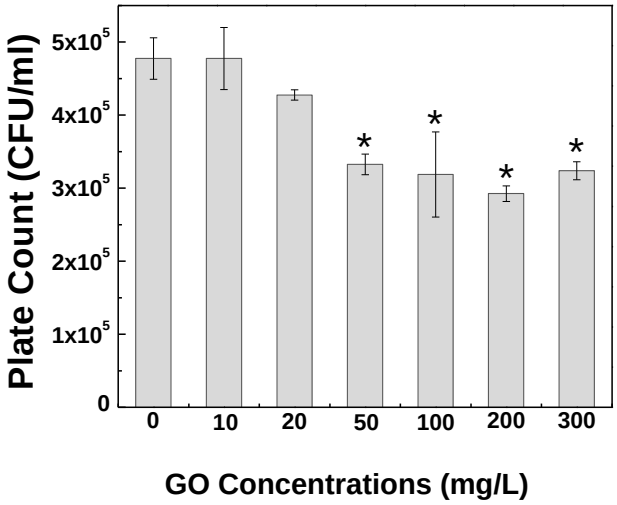

Figure 2. Enumeration of total viable bacterial cells (CFU/ml) in activated sludge after 5 h incubation with different concentrations of GO. ***** refers to statistically significant different results between control and the corresponding sample. Control sample does not contain any nanomaterial.

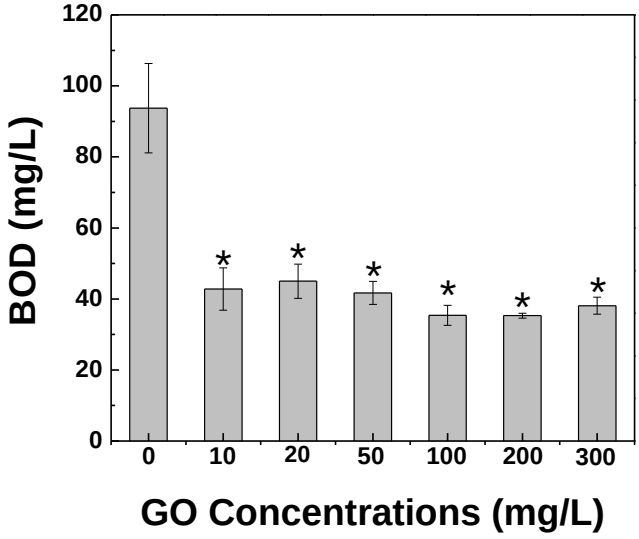

Figure 3. 5-day BOD results of the activated sludge samples with different concentrations of GO. ***** refers to statistically significant different results between control and the corresponding sample. Control sample does not contain any nanomaterial.

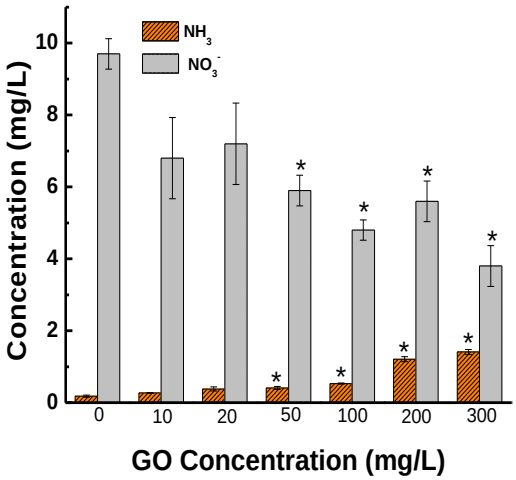

Figure 4. Concentrations of NH_3-N and NO_3^-, measured in activated sludge samples incubated with different GO concentrations. * refers to statistically significant different results between control and the corresponding sample. Control sample does not contain any nanomaterial.

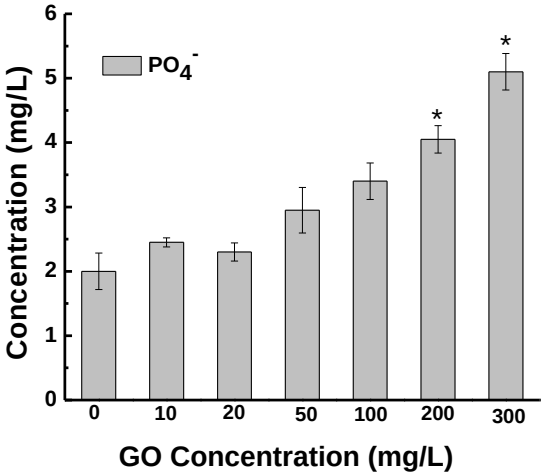

Figure 5. Concentrations of PO_4^-, measured in activated sludge samples incubated with different GO concentrations. * refers to statistically significant different results between control and the corresponding sample. Control sample does not contain any nanomaterial.

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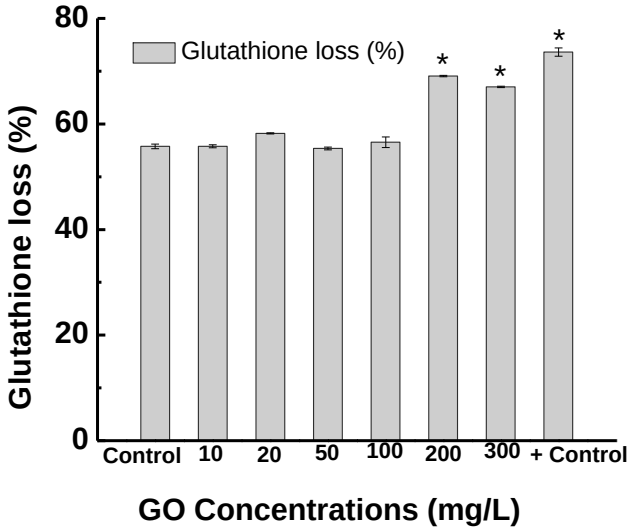

Figure 6. Loss of Glutathion as an indicator of ROS production. * refers to statistically significant different results between control and the corresponding sample. Control sample does not contain any nanomaterial. + Control contains H_2O_2 as oxidizing agent

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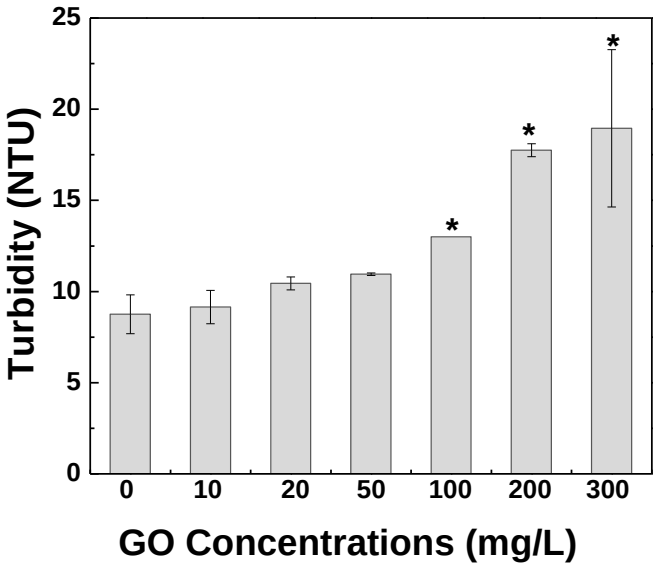

Figure 7. Turbidity (NTU) of the supernatant of activated sludge samples after 2 h settling period.* refers to statistically significant different results between control and the corresponding sample. Control sample does not contain any nanomaterial.

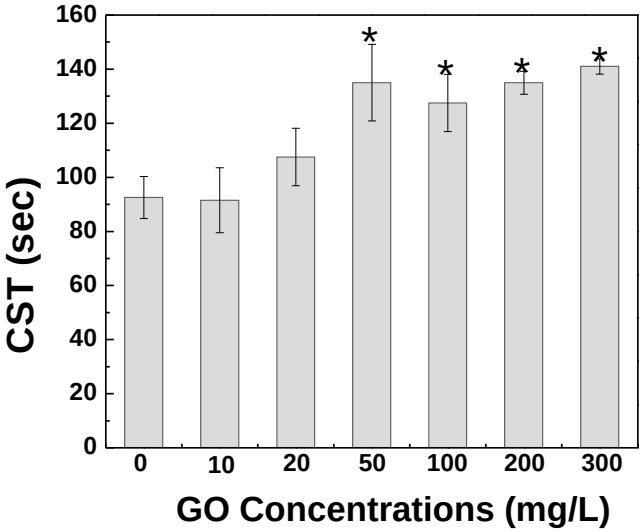

Figure 8. Capillary suction time (CST) required for dewatering of settled activated sludge samples after 2 h settling period. * refers to statistically significant different results between control and the corresponding sample. Control sample does not contain any nanomaterial.

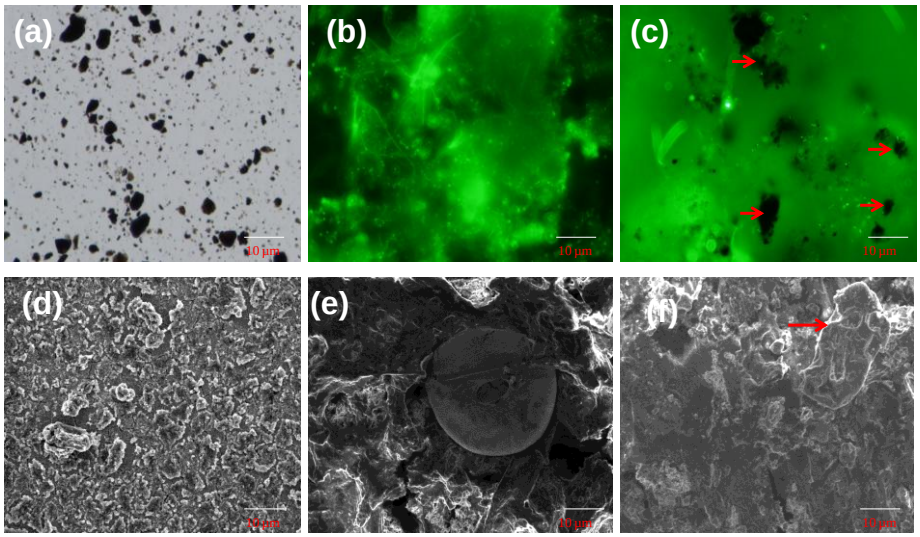

Figure 9. (a) Bright field image of aqueous suspension of GO; (b) fluorescence image of control activated sludge (no GO); (c) fluorescence image of activated sludge with GO; (d) SEM image of aqueous suspension of GO; (e) SEM image of control sludge (without GO; (f) SEM image of activated sludge with GO. Fluorescence and SEM imaging were done at 40x and 1000x magnifications, respectively. Red arrow sign indicates GO sheets in sludge flocs.

Supplementary Material

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